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Green and Sustainable Technology for High-Efficiency and Low-Damage Manipulation of Densely Crosslinked Proteins

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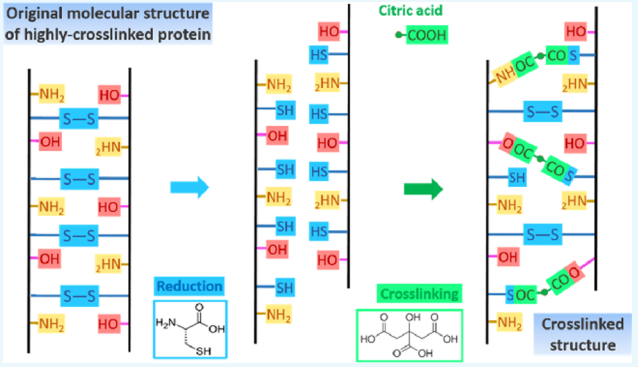
Supporting Information

ABSTRACT: A two-step technology using nontoxic and eco-friendly chemicals is developed for the durable setting of densely/highly crosslinked proteins, such as wool and hair. Currently, most technologies for morphological modification are effective only for materials from non-highly-crosslinked proteins and cellulose. Before their morphological change, only water is needed to interrupt hydrogen bonds and ionic linkages, which stabilize the relative positions of molecules in non-highly-crosslinked proteins and cellulose. However, highly crosslinked proteins contain disulfide crosslinks, which are insusceptible to water. Thus, the controlled cleavage of disulfide bonds is required for creating new morphologies of highly crosslinked protein materials, such as hair and wool. Herein, cysteine and citric acid (CA) were used for the two-step setting of highly crosslinked proteins. This recipe showed better morphological change and less mechanical loss than commercial hair styling products. A reaction between CA and keratin was proposed, and verified via NMR and Raman spectra and titration. This technology could be a prospective alternative to achieve durable hair setting, anticrease finishing of wool textiles, and other durable morphological changes needed for highly crosslinked proteins.



INTRODUCTION

The durable or semidurable setting of natural macromolecules, as an effective approach to modify the appearance and performance properties of macromolecular materials, has been a topic attracting growing attention from both academic and industrial scientists.[1,2] For example, in the textile industry, cotton, silk, and wool textiles should be set for antiwrinkle or durable-press effects;[3] and in the cosmetic industry, hair should be set for durable and semidurable styling purposes.

Currently, most processing technologies targeting durable or semidurable morphological changes are effective for materials based on non-highly-crosslinked proteins and cellulose, but not for materials based on highly/densely crosslinked proteins. The difference stems from the disparity between their molecular structures. To ensure the newly formed morphologies are durable, intermolecular interactions between the molecules in the original proteinous or cellulosic materials should be disrupted. In non-highly-crosslinked proteins and cellulose, hydrogen bonds and ionic linkages stabilize intermolecular positions, and can be easily interrupted by water molecules.[4] However, in highly crosslinked proteins, disulfide bonds that dominate the intermolecular interactions are insusceptible to water,[5] and thus render conventional setting methods for non-highly-crosslinked proteins and cellulose ineffective. Without breaking the strong internal disulfide crosslinks among keratin molecules, new morphologies could not be effectively formed by merely applying external crosslinks. Therefore, the controlled cleavage of disulfide bonds became the premise of effective setting of new morphologies of highly crosslinked protein materials.[6] So far, not much work has been reported on the high-efficiency setting of wool textiles or human/animal hair using only crosslinking steps under mild conditions. Hair setting was taken as a typical example to verify the effectiveness of the two-step process. In the first step, hair was chemically reduced by reductants, typically thioglycolates, to break the intermolecular disulfide bonds, rendering the keratin molecules capable of sliding by each other.[7] In the second step, the reduced hair was treated with hydrogen peroxide to rebuild the broken disulfide bonds, or formaldehyde to crosslink the keratin molecules in their new positions, to set the designated hair styles.

However, these chemicals are usually toxic or environmentally hazardous, and could easily contact the scalp during operation. Thioglycolates, including acetate thioglycolate and ammonium thioglycolate, usually at around 8% in the first step perming solution,[5] are toxic, carcinogenic, erosive, and

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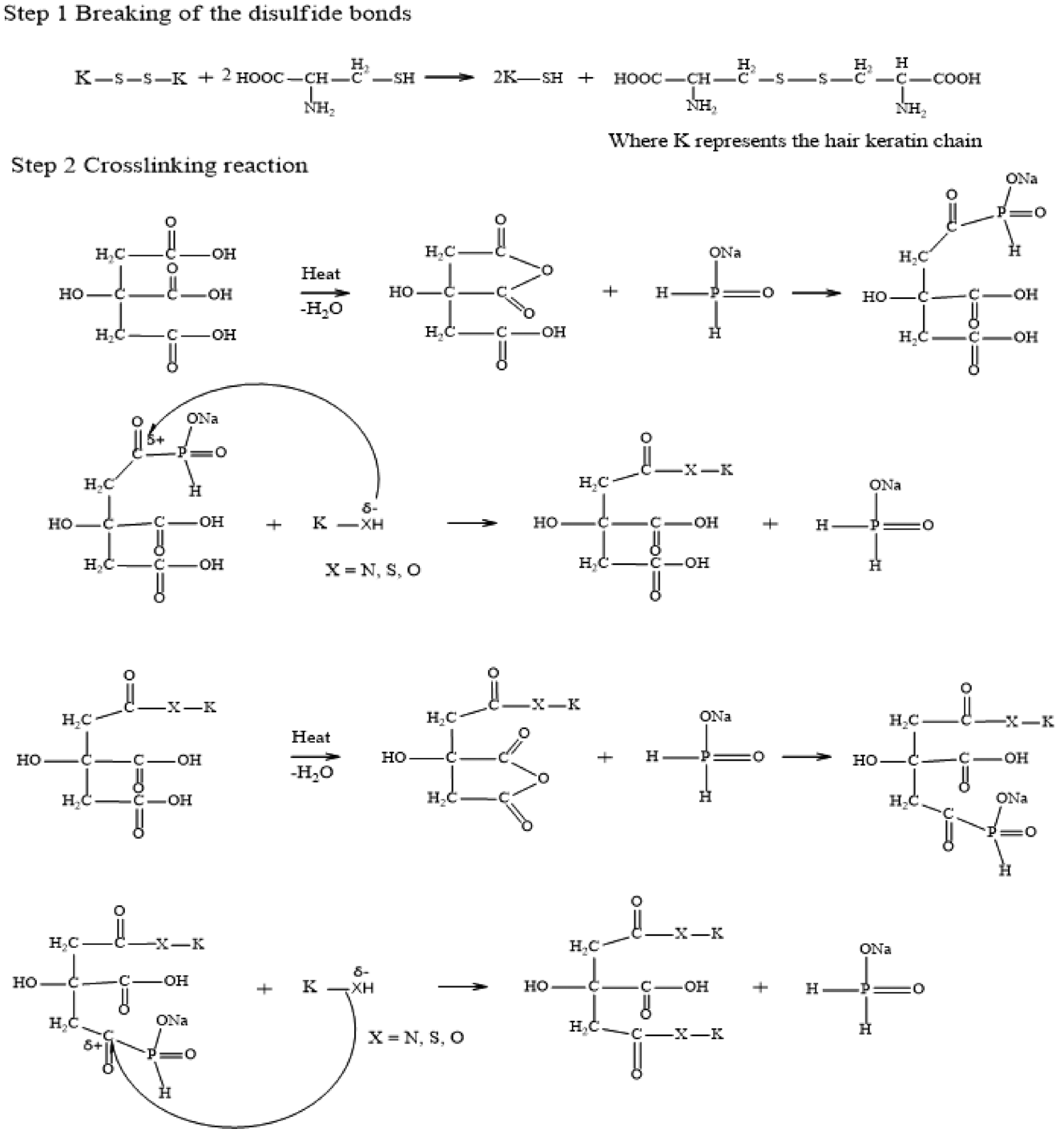

Figure 1. Scheme of hair setting reactions, including reduction of hair and crosslinking between hair and CA.

flammable with potential to generate poisonous hydrogen sulfide gas.[8,9] A number of occupational health problems, especially the impairment of reproductive systems, have increasingly been reported.[10] Hydrogen peroxide for hair setting could cause irritation to eyes, skin, throat, and respiratory airway.[11] Moreover, formaldehyde was usually used at concentrations higher than the allowed upper limit,[12,13] and has caused the most occupational health problems worldwide.[14−16]

Therefore, safer and greener chemicals are needed for hair setting and other treatment for highly crosslinked proteins. Cysteine and citric acid (CA) could be optimal candidates for the first-step cleavage of disulfide bonds and second-step rebuilding of new disulfide bonds, respectively, for stabilization of new morphology. Cysteine is a standard amino acid with a thiol group, and thus, has strong reducibility.[17] Application of the environmentally friendly reductant could meet the requirements for sustainable development of material industry.[18] CA is fermented from corn or potato starch on an industrial scale.[19] More than 66% of the production of CA is used in beverages and food processing, indicating the general safety of CA.[20]

Cysteine has shown effectiveness in reducing the disulfide bonds in hair keratin[21] in the first step of hair styling. However,

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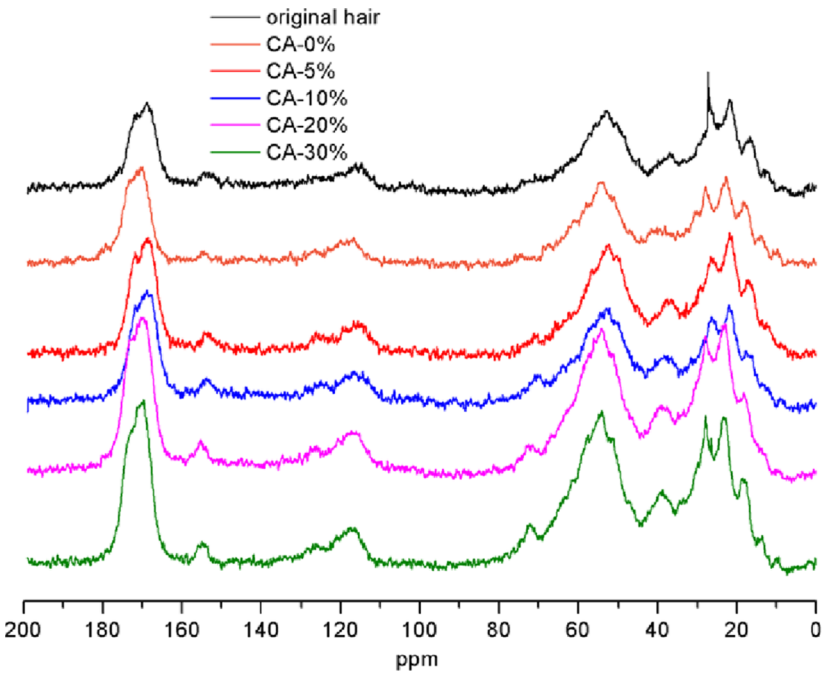

Figure 2. ^{13}C CP/MAS NMR spectra of original hair, CA-0% group (air oxidized hair, hair treated under the same conditions without using CA), and hair crosslinked with 5, 10, 20, and 30% CA (reduction: 5% cysteine based on the weight of hair, 2 M urea, pH 9.5; crosslinking: 180 °C, 4 min).

in some research, cysteine was reported to be less effective than thioglycolic acid for hair perming, due to poorer penetration into the hair cortex.[22,23] However, it could be assumed that with slower reaction with hair, the loss of hair strength as a side effect of the reduction could be better controlled. Furthermore, as has been proven, with extra swelling of hair, cysteine reduction could substantially enhance the reduction efficiency,[24] and subsequently, setting efficiency.[25]

Nontoxic CA crosslinking has the potential to be used in the second step of hair styling to rebuild the disulfide bonds at new positions to restore the mechanical robustness of hair.[26,27] CA crosslinking of protein fibers and protein nanoparticles remarkably improved the linkages improving the performance properties, such as the tensile properties and water stability.[28−30] Furthermore, in vitro assessment proved that the CA crosslinking of proteins is cytocompatible.[31] However, to the best of our knowledge, there has been no report regarding the crosslinking of hair keratin using CA for durable hair styling.

In this research, green and nontoxic chemicals, cysteine as a reductant combined with swelling agent urea, were used for reduction, and CA subsequently was used for crosslinking in hair setting. The setting efficiency, as well as the dry and wet tensile properties of hair treated with the nontoxic approach were compared with those of hair treated with commercial products. Reduction and crosslinking were quantitatively analyzed via titration to verify their occurrence. The reaction between CA and hair keratin was characterized by NMR and Raman spectroscopy.

RESULTS AND DISCUSSION

Reaction Mechanism and Crosslinking Degree. The detailed reaction steps of the reduction of hair keratin using cysteine and the crosslinking between hair keratin and CA are demonstrated in Figure 1. As shown in step 1, the −SH in cysteine exchanged with the −S−S− in keratin and weakened the interaction among the keratin, thus rendering the keratin molecules capable of sliding alongside each other.

After endowing new shapes to the reduced hair, CA was added to create new intermolecular bonds and fix the new morphology of the hair. The possible reaction steps between CA and hair keratin catalyzed with SHP are shown in step 2. Under temperatures as high as 180 °C, two carboxyl groups could react to form an anhydride, releasing one molecule of water. Subsequently, acylation between the anhydride and SHP produces a molecule of intermediate (1), which could be substituted by nucleophilic groups, such as −OH, −SH, and $-NH_2$, in the reduced keratin, generating an ester group (2) and releasing a molecule of SHP and a carboxyl group in the CA. Till now, one carboxyl group in the CA has formed an ester group with hair keratin and two are still available for reaction. The two carboxyl groups could again form another ester group with another nucleophilic group in the same keratin molecule or another keratin molecule. The reactions lead to intramolecular and intermolecular crosslinking. Regarding that in one CA molecule, only one carboxyl group participates in the reaction with keratin, the two other free carboxyl groups and the hydroxyl groups could then form hydrogen bonds with amine groups, hydrogen groups, and carboxyl groups in keratin molecules to enhance the intermolecular or intramolecular interactions. The newly formed ester groups are stable, whereas the hydrogen bonds might be easily destroyed under wet conditions.

NMR. Figure 2 shows the NMR spectra of the original hair, air oxidized hair, and hair crosslinked with CA with concentrations of 5, 10, 20, and 30%. All of the CA crosslinked hair had obvious peaks at 74 ppm, indicating the presence of carbon atoms, which are connected to the hydroxyl groups in the CA molecules,[3] whereas the original hair and air oxidized hair did not. The difference suggests the occurrence of a reaction between the hair and CA in the treated hair. The peaks between 170 and 180 ppm representing carbonyl groups in

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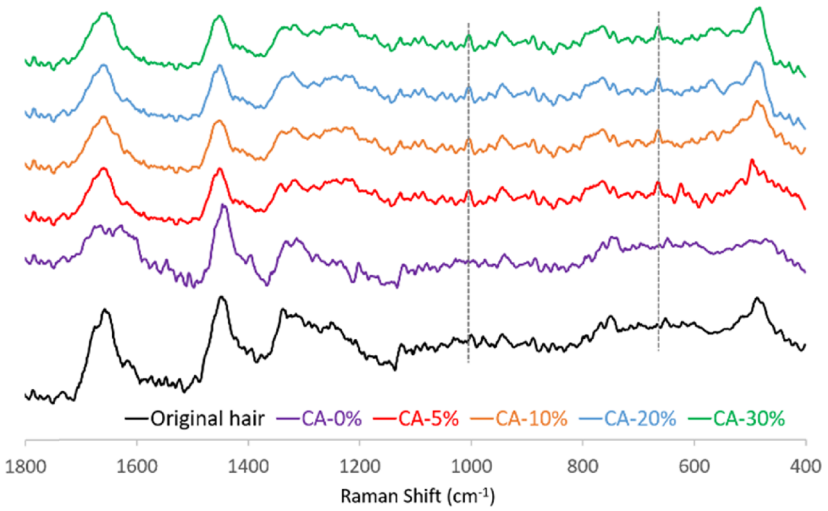

Figure 3. Raman spectra of cortex section (15 μm under the fiber surface) of original hair, CA-0% group (air oxidized hair, hair treated under the same conditions without using CA), and hair crosslinked with 5, 10, 20, and 30% CA (reduction: 5% cysteine based on the weight of hair, 2 M urea, pH 9.5; crosslinking: 180 °C, 4 min). The dot lines indicating peaks at around 1000 and 650 cm^{-1} represent the cysteine-S-sulfonate bonds (−S−O−) and thioester groups (−COS−), respectively.

keratin existed in all of the groups of hair.[34] However, among all of the sample groups, there was no significant difference in the intensity of the carbonyl peak. This could be because, compared to the number of carbonyl groups in original hair keratin, the amount of carbonyl groups increased due to reacted CA molecules was negligible.

Raman Spectra. In Figure 3, the peak at around 650 cm^{-1}, indicating formation of thioester group (−COS−),[35−37] existed only in the spectra of hair crosslinked with CA at concentrations of 5−30%, but not in that of original hair and air oxidized hair. It could be inferred that the −COOH groups in CA reacted with the −SH groups in reduced hair keratin. Moreover, the peak at around 1000 cm^{-1},[21,38,39] representing cysteine-S-sulfonate bonds (−S−O−) most probably in cysteic acid, could be observed in the 5−30% CA crosslinked hair cortex spectra, and not in that of the cortex of untreated and 0% CA treated hair. This sulfur−oxygen bond was also formed via oxidation of sulfhydryl groups in reduced keratin. Both peaks of 650 and 1000 cm^{-1} inferred that crosslinking with CA might boost oxidation of sulfhydryl groups. However, the mechanism remains unclear.

Efficiency of Nontoxic Hair Setting. Hair is composed of about 80% keratin. The morphology of hair is stabilized due to fixation of the relative positions of keratin molecules by disulfide crosslinking, which is attributed to the existence of about 18% of cysteine in its amino acid composition.[5] To modify the morphology of hair, disulfide bonds should be cleaved to allow keratin molecules to slide alongside each other under external force. After new shapes are induced, the intermolecular interactions among keratin should be re-established.

All chemicals employed in the reduction and crosslinking for hair perming were nontoxic. In the first step, urea was used to break the hydrogen bonds, salt linkages, and Van der Waal's force, thus swelling the hair with tight structures. Under this circumstance, the reductant, cysteine, could penetrate the interior of the hair. With pH adjusted to about 9, cysteine has a strong reducing capability, and could effectively break the disulfide bonds throughout the entire hair. In the second step, new covalent bonds between carboxylic groups in CA and functional groups, such as amine groups, hydroxyl groups, and thiol groups in keratin could be created. Consequently, the keratin molecules in their new positions, and subsequently, the new morphologies of hair were stabilized.

Figure 4 demonstrates the effect of the concentration of CA on perming efficiency (PE) after using the green approach, and

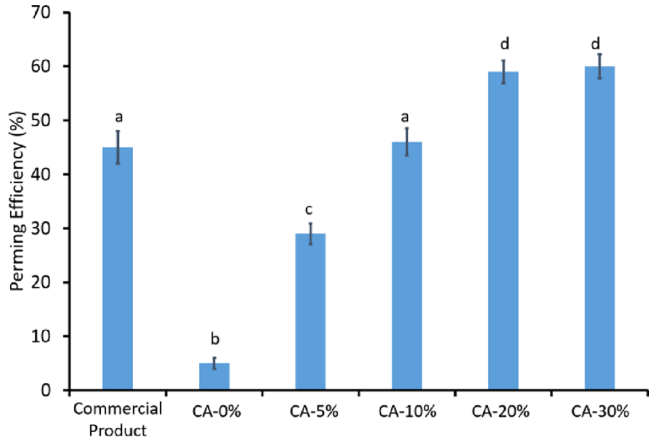

Figure 4. Effect of CA concentration on PE using CA as the crosslinking agent in the second step. A commercial perming product with the highest PE among 10 was selected as the control. A CA-0% group (air oxidized hair, hair treated under the same conditions without using CA) (reduction: 5% cysteine based on the weight of hair, 2 M urea, pH 9.5; crosslinking: 180 °C, 4 min. Commercial products were applied following the suppliers' instructions.) Above each bar, different letters "a"−"d" are labeled to indicate significant differences among the data points.

comparison with commercial perming product. The average PE increased remarkably from 5 to 60% as the concentration of CA increased from 0 to 20%, but did not increase as the concentration of CA increased to 30%. CA-0% groups indicate that the hair was oxidized in air and no external crosslinking was applied. The commercial product using hydrogen peroxide oxidation led to a PE of 45%, similar to the result of perming using 10% CA in crosslinking.

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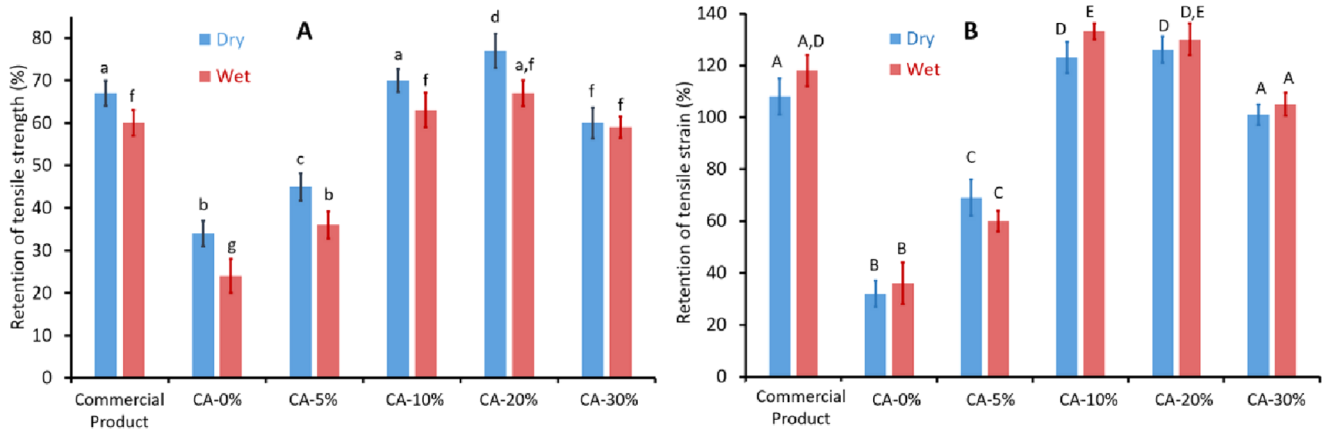

Figure 5. Percent retention of dry and wet tensile strength (A) and elongation (B) of hair fibers crosslinked with CA at different concentrations. CA-0% group (air oxidized hair, hair treated under the same conditions without using CA) (reduction: 5% cysteine based on the weight of hair, 2 M urea, pH 9.5; crosslinking: 180 °C, 4 min). Above each bar, different letters a–f and A–E are labeled to indicate significant differences among the data points.

The perming results were highly affected by the amounts of functional groups that participated in building new covalent bonds during crosslinking or oxidation. When the CA concentration was 0, only oxygen in the air could oxidize the sulfhydryl groups into disulfide bonds. However, the oxidation was insufficient to transform all of the sulfhydryl groups. By introducing CA in the second step of hair perming, extra connections among keratin molecules via re-formation of the thioester, ester, and amino groups between −COOH groups in CA and the $-NH_2$, −OH, and −SH groups of reduced keratin, in addition to the disulfide bonds formed due to air oxidation. The maximum PE% reached at a CA concentration of 20% might be due to occupation of all of the functional groups in keratin molecules, rendering further addition of CA into the system ineffective. Changes in the amount of the functional groups −COOH, −SH, and $-NH_2$ were quantified to verify this assumption.

Mechanical Properties of Hair Fibers after Perming. Figure 5 shows that with similar PE, the % retention of dry and wet tensile strength and strain of hair treated by the commercial product were both lower than that of hair treated by 10% CA. The better retained mechanical properties of hair could be due to the more effective recovery of intermolecular interactions induced by CA crosslinking, compared to hydrogen peroxide oxidation. Figure 5 also demonstrates that the average % retention of dry and wet tensile strength increased remarkably from 34 to 77% and from 24 to 67%, respectively, as the concentration of CA increased from 0 to 20%, and then decreased to 60 and 59%, respectively, as the concentration of CA increased to 30%. The average % retention of dry and wet tensile strain increased remarkably from 32 to 126% and from 36 to 130%, respectively, as the concentration of CA increased from 0 to 20%, and then decreased to 101 and 105%, respectively, as the concentration of CA increased to 30%. Compared to the dry state, wet tensile strength was always lower, whereas wet tensile strain was always higher. The difference between dry and wet hair was acceptable, and was attributed to interruption of the hydrogen bonds among the keratin backbones by water molecules.

The tensile strength and strain of hair decreased and could not fully recover after the cleavage of the disulfide bonds. Rebuilding of the disulfide bonds and other intermolecular forces by either oxidation or crosslinking could recover the tensile properties of hair with variable recovery extents. Air oxidation played a major role when 0% CA was used in the second step of perming. The resultant % retention of tensile properties was low due to a low re-establishment of crosslinking. As more CA was added for hair perming, additional intermolecular bonds, including thioester, ester, and amino groups between −COOH groups were formed in CA and $-NH_2$, −OH, and −SH groups in reduced keratin. Therefore, the tensile properties of the hair fibers could be more effectively recovered. However, a further increase in CA concentration to 30% decreased the tensile strength and strain due to over-crosslinking.[40]

To verify the reduction and crosslinking reactions, and effect of crosslinking on the PE, the changes in the amounts of −SH, $-NH_2$, and −COOH on hair keratin are shown below. However, due to limitations of the characterization approaches, change in the −OH amount, which was also important for the reaction, was not measured in this research.

Change in the Amounts of Functional Groups in Hair before and after Setting. In Figure 6, the concentration of sulfhydryl groups in hair increased from 17 μmol g^{-1} to nearly 238 μmol g^{-1} after reduction using cysteine. With cysteine reduction and air oxidation (CA-0%), the −SH concentration significantly decreased to 168 μmol g^{-1}. Oxygen in the air oxidized about 30% of the total sulfhydryl groups into disulfide groups. The corresponding PE, percentage retention of tensile strength, and percentage retention of tensile elongation were 5, 34, and 36%, respectively. The insufficient perming effectiveness was due to the insufficient reconnection of disulfide bonds of air oxidation.

By increasing the CA concentration to 30%, the −SH concentration gradually and remarkably dropped to 22 μmol g^{-1} (20% CA) and 19 μmol g^{-1} (30% CA), which is very close to the amount of −SH in the untreated hair. The corresponding PE, % retention of tensile strength, and % retention of tensile elongation increased to 59, 77, and 126%, respectively. It could be inferred that with the addition of 20% or more CA, a majority of −SH groups generated from cysteine reduction could participate in the reactions. The results verified that reconnection of intermolecular linkages induced by CA crosslinking played a major role in the setting of hair.

Figure 7 indicates that as the CA concentration increased, the amount of carboxyl groups in the hair gradually increased from

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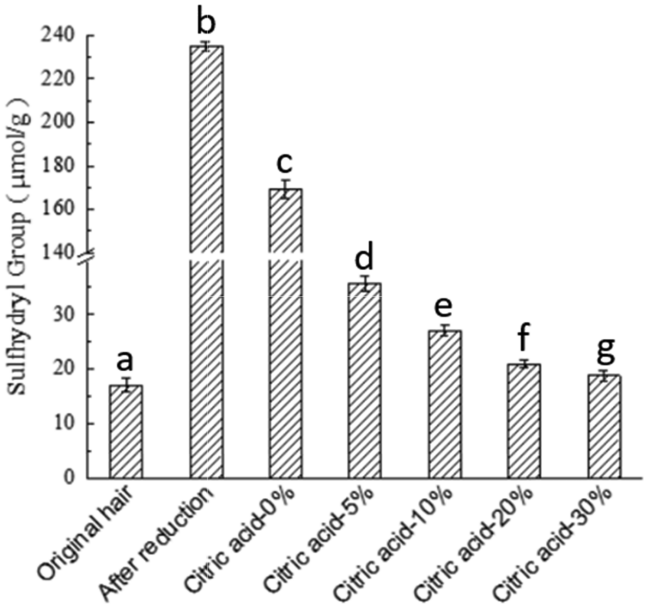

Figure 6. Concentration of sulfhydryl group (−SH) in untreated hair, CA-0% group (air oxidized hair, hair treated under the same conditions without using CA), and hair permed with 5, 10, 20, and 30% CA. Above each bar, different letters a−g are labeled to indicate significant differences among the data points.

5 to 32 mmol g^{-1}. Not all three carboxyl groups in one CA molecule could react with the functional groups in keratin due to steric hindrance, leaving two or one carboxyl groups unreacted. Figure 4B shows the amount of amine groups gradually decreased from 1.6 to 0.7 mmol g^{-1}.

When the CA concentration was 0, the amount of carboxyl and amine groups did not change significantly, as shown in Figure 7. It could be inferred that the amine groups did not participate in the reactions of reduction and air oxidation, and the perming effect could be attributed only to the re-formation of disulfide bonds. As the CA concentration increased, the acylation reaction between amine groups and carboxyl groups led to a decrease in the amount of amine groups. When the CA concentration was 5, 10, and 20%, about 25, 44, and 56% of amine groups participated in the reaction, resulting in perming efficiencies of 27, 45, and 59%, respectively. As the concentration of CA further increased to 30%, the PE did not increase, probably because all of the amine groups capable of reacting with carboxyl groups in CA were consumed.

Application of the Nontoxic Hair Styling Product for Hair Straightening. High efficiency in straightening and good retention of mechanical properties of hair could also be observed when using the same nontoxic cysteine/CA hair styling product to straighten natural curly hair. Table 1 demonstrates that the straightening efficiency % and retention of mechanical properties of natural curly hair using the nontoxic cysteine/CA hair styling product were better, compared with

Table 1. Straightening Efficiency and Retention of Mechanical Properties of Natural Curly Hair Using the Nontoxic Cysteine/CA Hair Styling Product Compared with the Commercial Hair Straightening Product

property	condition		the best of the 10 commercial products (%)	nontoxic cysteine/CA product (%)
straightening efficiency (%)[a]	after straightening		80	83
	after 30 cycles of washing		60	70
retention of mechanical properties (%)	dry	tensile strength	30	36
		tensile strain	90	95
	wet	tensile strength	40	39
		tensile strain	105	119

[a]Straightening efficiency % = length after straightening treatment/straighten length of curly hair before treatment × 100%.

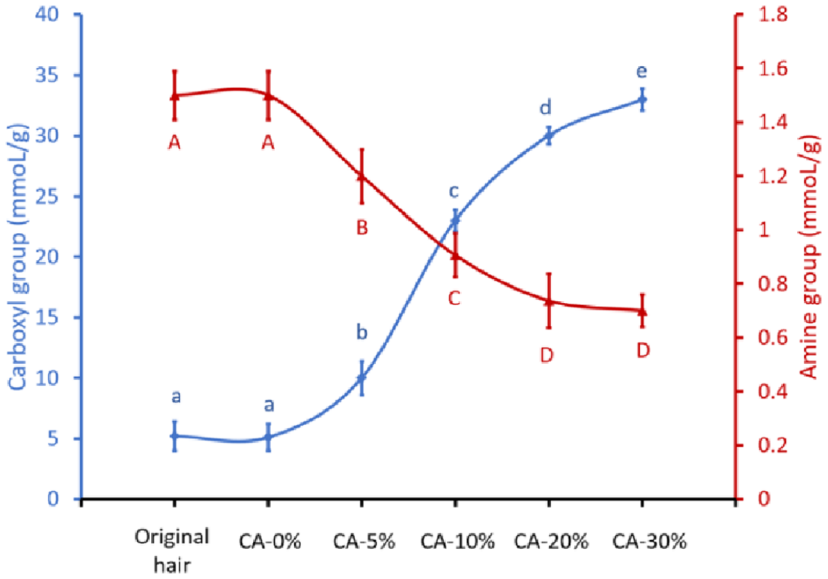

Figure 7. Concentration of −COOH and $-NH_2$ in untreated and CA crosslinked hair samples. CA-0% group (air oxidized hair, hair treated under the same conditions without using CA) (reduction: 5% cysteine based on the weight of hair, 2 M urea, pH 9.5; crosslinking: 180 °C, 4 min). Different letters a−e and A−D are labeled to indicate significant differences among the data points.

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the best of the 10 commercial hair straightening products. The commercial product used a relaxer in the first step and neutralizer in the second step of hair straightening. The alkaline solution used in the first step had a pH at around 13 and caused hydrolysis of the keratin backbones as well as disulfide bonds. The neutralization in the second step could mainly help rebuild the disulfide bonds. On the other hand, in the nontoxic cysteine/CA formula, the keratin backbones were not as severely damaged due to the much lower pH in the first step. In addition to the rebuilding of the disulfide bonds, CA could effectively react with −SH, −OH, −COOH, and $-NH_2$ groups in the keratin, leading to more durable straightening results and stronger hair fibers.

CONCLUSIONS

To manipulate highly crosslinked proteins, cysteine and CA, two naturally occurring chemicals that could be fermented from dextrose and starch, respectively, were used for the reduction and crosslinking of proteins, respectively. Hair styling was used as an example of setting of highly-crosslinked proteins. Cysteine was effective in breaking the disulfide bonds, and this was indicated by the significantly increased amount of sulfhydryl groups after reduction. CA was efficient in crosslinking keratin, which was indicated by the changes in the amounts of −SH, −COOH, and $-NH_2$ groups. The reaction between CA and hair was verified using solid-state NMR. The peak at 74 ppm only present in the spectra of the CA crosslinked hair indicated a carbon atom connecting with the hydroxyl group in CA. The Raman spectra of CA crosslinked hair also showed peaks at 650 cm^{-1}, representing thioester groups, which formed between the −SH groups in the reduced hair and −COOH groups in CA. Compared to the commercial products using hazardous chemicals, our technology showed higher curling/straightening efficiencies and better retention of mechanical properties of hair. In summary, this two-step technology could be promising for achieving durable hair setting, anticrease finishing of wool textiles, and other morphological changes for highly crosslinked proteins.

MATERIALS AND METHODS

Materials. Long straight hair with a length of around 30 cm was collected from a 30 year old Asian female. Natural curly hair with length of around 20 cm was collected from a 50 year old African American female. Cysteine, sodium carbonate, CA, sodium hypophosphite (SHP), ethylenediaminetetraacetic acid (EDTA), and glycerol were purchased from EMD Chemicals Inc., Gibbstown, NJ. Urea was purchased from Oak Chemical, Inc., West Columbia, SC. 5,5′-Dithiobis-(2-nitrobenzoic acid) (DTNB) was purchased from G-Biosciences, St. Louis, MO. The purity of the chemicals was considered in all the calculations of concentrations. SHP is generally regarded as safe (GRAS) by the FDA and has been used as a food additive for decades.[41,42] Cysteine is safe and has been widely used in personal care products, including cosmetics, for decades.[43,44] Urea has been accepted as an effective swelling agent for keratin materials.[45,46]

Hair Setting. Before setting, the hair was shampooed, rinsed, and dried. Hair setting included two steps, swelling/reduction and crosslinking. The straight hair was rolled up onto a glass rod to obtain curls. In the first reduction step, the hair was treated with an aqueous reduction solution composed of 10% of the freshly prepared cysteine solution in 2 M urea solution with pH adjusted to 9.5 using sodium carbonate. The use of 2 M urea solution was selected after careful initial experiments. Using a higher concentration of urea caused the hair to be destroyed easily due to accidentally prolonging step 1; while using a lower concentration meant the hair could not be effectively swollen to facilitate the necessary reduction of disulfide bonds in the interior hair fibers, leading to ineffective perming of the hair (data not shown). The reduced hair was rinsed with distilled water three times at an infinite liquor ratio at room temperature, and then heated at 80 °C until the weight became constant. The crosslinking agent containing 5, 10, 20, and 30 wt % CA was prepared with SHP at 50 wt % CA. The hair on the rod was soaked in the crosslinking solution and oscillated in an ultrasonic bath for 10 min. The hair was dried at 80 °C for 10 min. The soak−dry procedure was repeated several times until the weight became constant. The final pick-up of crosslinking solution on the hair was controlled in the range of 90−100 wt %, based on the dry weight of the hair bundle. At last, the hair bundles were heated at 180 °C for 4 min to conduct the crosslinking. Straightening followed the same procedures and recipes, except that the hair was straightened under tension instead of curled on rods.

PE was calculated based on the change in hair length before and after perming treatment, as shown in eq 1.

$$\text{perming efficiency} = \frac{\text{number of loops after perming/fiber length after perming}}{\text{number of loops before perming/fiber length before perming}} \times 100\% \quad (1)$$

After curling or straightening, the hair was rinsed in distilled water at room temperature three times, wiped with a paper towel to remove free water, and dried under the environmental conditions of 21 °C and 65% relative humidity for at least 24 h before any measurement or testing. Hair perming and straightening basically used the same procedures. Using the nontoxic cysteine and CA, perming of straight hair was discussed in detail, followed by the straightening results of naturally curly hair.

Tensile Properties. The fineness of the hair fibers was measured in terms of denier, which is the weight of 9000 m of fibers in grams. The weight of known lengths of hair fibers was measured to calculate the denier of the fibers. Before testing the dry tensile properties, the hair was first conditioned to determine the wet tensile properties, the hair was soaked in water for at least 30 min before testing. The tensile properties of the fibers in terms of breaking tenacity and breaking elongation were tested using an Instron tensile testing machine (model 4400; Norwood, MA) according to ASTM standard D-3822. In the test, a gauge length of 1 in. and crosshead speed of 18 mm min^{-1} were used. For each condition, about 30 specimens were tested for each fiber sample. The wet strength of keratin fibers was determined immediately after immersing the fibers in water at room temperature for 30 min.

Titration of Sulfhydryl Groups, Carboxyl Groups, and Amine Groups. To verify the reaction between hair keratin and CA, the change in the amounts of −SH, −COOH, and $-NH_2$ groups was determined using titration. A solid-phase assay for total thiol group content was carried out according to the colorimetric reaction method described by Yoshimizu et al.[32] About 30 mg of ground hair was suspended in 1.0 mL of reaction buffer consisting of 8 M urea, 10 mM DTNB, 3 mM EDTA, and 0.2 M Tris−HCl, pH 8.0. Samples were incubated at room temperature in a N_2 atmosphere for 15 min, and then

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centrifuged at 9000 rcf for 10 min. The absorbance of the supernatant at 412 nm was determined using a UV/vis spectrophotometer (model DU 720; Beckman Coulter Inc. Brea, CA). The concentration of thiol groups was calculated based on eq 2. Three replica experiments were conducted for each data point.

$$\text{thiol group content}\left(\frac{\text{mmol}}{\text{g}}\right) = \frac{\left(\frac{A}{13\,600}\right) \times V}{m} \times 100\% \quad (2)$$

wherein A is the value of absorbance, V is the volume of reaction buffer, and m is the weight of hair samples.

Based on our previous study,[33] carboxyl group and amino group content in the hair sample were determined via titration using a Mettler Toledo SevenMulti S47 pH/conductivity meter equipped with an Inlab Expert Pro electrode and an Inlab 730 probe for pH and conductivity measurements, respectively (Mettler Toledo, Columbus, OH). About 1 g of ground and delipidized crosslinked and original hair sample was precisely weighed, and dispersed in 20 mL of standardized 0.05 mol L^{-1} HCl, in which the carboxylic and amino groups in the hair sample were protonated. Standardized 0.05 mol L^{-1} NaOH solution was used. Conductivity and pH values were recorded after addition of about 0.2 mL NaOH solution each time. Three replica experiments were conducted for each data point.

Raman and NMR Spectra. Raman and solid-state ^{13}C NMR spectroscopy were used to detect new groups to verify the occurrence of a reaction between hair keratin and CA. The hair samples were embedded in epoxy resin, cured, and then microtomed to a thickness of 1.5 μm, and mounted on slide glasses. All Raman spectra were recorded 20 μm under the surface of hair on a Raman spectroscope (inVia H 18415; Renishaw, Illinois, IL). The laser excitation was provided by an argon ion laser at a cross slit of 100−200 μm with a power of 30−50 mW at a wavelength of 632 nm. Solid-state ^{13}C NMR was performed on a Bruker Avance 600 MHz NMR Spectrometer (Bruker BioSpin, Billerica, MA). Each keratin sample was scanned for 8 h to obtain good signals.

Statistical Analysis. All of the data points were compared using the one-way analysis of variance with the Scheffé test with a confidence interval of 95%. A p value smaller than 0.05 indicated a statistically significant difference. Standard deviations are shown by the error bars in the figures, and the data in the figures labeled with different numbers or characters indicate significant differences among them.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsomega.7b00154.

Comparison of hazards of active ingredients in the commercial product and our product (Table S1) (PDF)

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